nature medicine

Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA

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Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major human pathogen. Traditionally, MRSA infections occurred exclusively in hospitals and were limited to immunocompromised patients or individuals with predisposing risk factors. However, recently there has been an alarming epidemic caused by community-associated (CA)-MRSA strains, which can cause severe infections that can result in necrotizing fasciitis or even death in otherwise healthy adults outside of healthcare settings[1,2]. In the US, CA-MRSA is now the cause of the majority of infections that result in trips to the emergency room[3]. It is unclear what makes CA-MRSA strains more successful in causing human disease compared with their hospital-associated counterparts. Here we describe a class of secreted staphylococcal peptides that have a remarkable ability to recruit, activate and subsequently lyse human neutrophils, thus eliminating the main cellular defense against *S. aureus* infection. These peptides are produced at high concentrations by standard CA-MRSA strains and contribute significantly to the strains' ability to cause disease in animal models of infection. Our study reveals a previously uncharacterized set of *S. aureus* virulence factors that account at least in part for the enhanced virulence of CA-MRSA.

Among several putative determinants of CA-MRSA virulence, the Panton-Valentine leukocidin (PVL) has received the most attention[4–7]. More than 20 years ago, it was suggested that this lytic toxin functions as a virulence factor in cutaneous infection[8,9], and results from a mouse infection model have indicated that purified PVL or heterologous overexpression of PVL contributes to the development of experimental necrotizing pneumonia caused by laboratory strains of *S. aureus*[10]. This type of disease is rare among CA-MRSA infections (less than 2% of cases)[11], and whether or not PVL contributes to the necrotizing pneumonia caused by CA-MRSA remains to be determined. In contrast, results obtained with CA-MRSA–isogenic PVL deletion strains indicate that PVL does not have a significant role in CA-MRSA skin and soft-tissue infections, which represent the most frequent disease manifestations of CA-MRSA, or in bacteremia[12]. Thus, the basic cause of CA-MRSA virulence remains undefined, prompting us to search for previously unidentified CA-MRSA virulence determinants.

We identified a group of peptides in *S. aureus* that are related in part to the phenol-soluble modulin (PSM) peptides described in *Staphylococcus epidermidis*. *S. epidermidis* PSM has been reported to elicit an inflammatory response in cells from a macrophage lineage[13], but its role in staphylococcal infection is poorly understood. By using analytical and preparative reversed-phase HPLC/electrospray mass spectrometry (RP-HPLC/ESI-MS), as well as N-terminal peptide sequencing (**Supplementary Fig. 1** online), we found that *S. aureus* secretes four shorter (~20 amino acids, which we define as the α-type) and two longer (~40 amino acids, which we define as the β-type) PSM-like peptides (**Fig. 1a**), whose genes are arranged in two gene clusters (**Fig. 1b**). In addition, *S. aureus* produces δ-toxin, which is similar to the α-type PSMs. Of note, the PSMα genes have not been previously described, owing to their lack of similarity to the PSM genes of *S. epidermidis* and to the fact that they are shorter than the threshold length for gene annotation.

To analyze whether PSMs are virulence determinants of CA-MRSA, we first compared the production of PSMs by representative hospital-associated (HA)- and CA-MRSA strains. Although the PSM genes are present in all sequenced *S. aureus* strains, we detected much higher *in vitro* PSM production in the most prevalent CA-MRSA compared to HA-MRSA (**Fig. 1c**), raising the possibility that PSMs contribute to the enhanced virulence of CA-MRSA[14]. To further investigate this hypothesis, we constructed isogenic gene deletion strains derived from the CA-MRSA strains MW2 (USA400)[4] and LAC (USA300)[5], in which the PSMα or PSMβ gene loci were deleted, or the *hld* gene was altered to abolish production of δ-toxin. RP-HPLC-ESI/MS analysis confirmed the specific absence of the particular PSM peptide(s) in the respective gene deletion strains (**Supplementary Fig. 1** online).

We investigated the virulence of the PSM deletion strains in comparison to the wild-type strains in mouse abscess and bacteremia models[12]. These models were selected on the basis of the prevalence of

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Received 26 March; accepted 21 August; published online 11 November 2007; corrected online 27 November 2007 (details online); doi:10.1038/nm1656

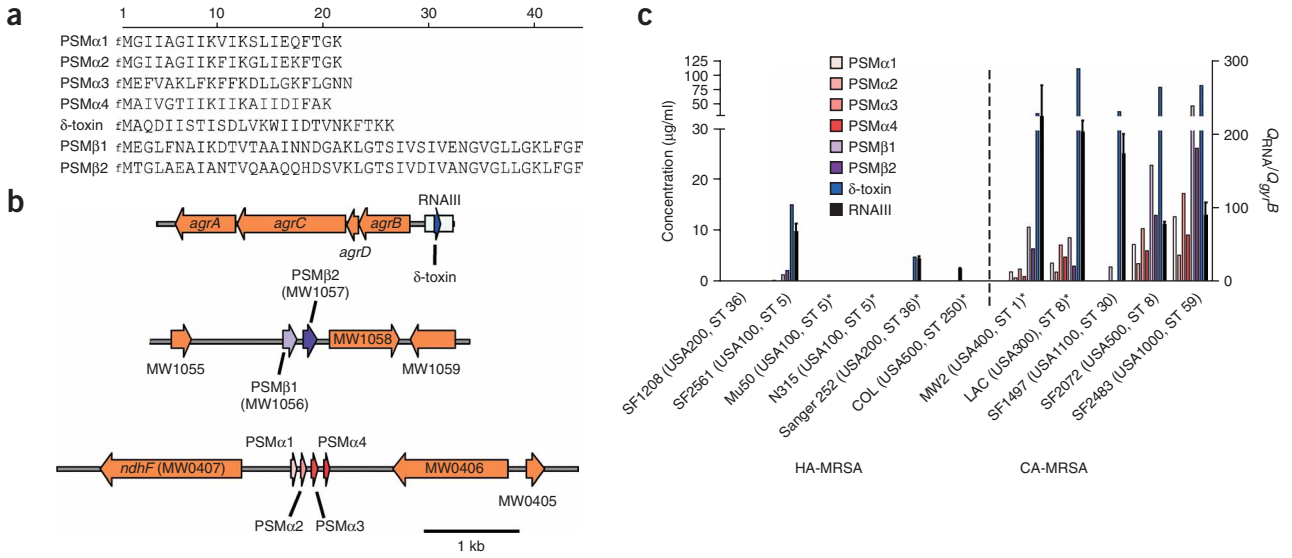

Figure 1 PSMs in *S. aureus*. (**a**) Amino acid sequences. PSMs are all formylated (f) at the N-terminal methionine residue. The bar at the top indicates length in amino acids. (**b**) Location of PSM genes in the genome of *S. aureus* MW2 (ref. 4). (**c**) Production of PSMs (as detected by RP-HPLC/ESI-MS in 8-h stationary-phase cultures) and of RNAIII (as detected by quantitative RT-PCR, in 4-h late exponential phase cultures, at maximal expression of *agr*) by standard CA- and HA-MRSA strains. Asterisk denotes *S. aureus* strain whose genome has been sequenced. *Q*, relative quantitation.

CA-MRSA in skin and soft-tissue infections[3] and severe sepsis[15,16]. MW2 (USA400), which typically causes sepsis in humans[15,16], was used for the bacteremia model, whereas LAC (USA300), by far the most prominent cause of community-associated skin and soft-tissue infections in the US[3], was used in the skin and soft-tissue infection model. In the bacteremia model, there was significantly reduced mortality in the mice infected with the PSMα deletion strain and, to a lesser extent, the δ-toxin–negative strain (**Fig. 2a**). Consistent with the sepsis data, the amount of the inflammatory cytokine TNF-α was significantly reduced in blood samples of mice infected with those mutant strains (**Fig. 2b**). Additionally, the LAC PSMα deletion strain, but not the other PSM deletion strains, had a significantly decreased ability to cause skin lesions in mice (**Fig. 2c,d**). Together, these data show that α-type PSMs have an essential function in the most important manifestations of CA-MRSA–induced disease.

To determine the mechanism by which PSM peptides promote virulence, we next tested the ability of these molecules to alter phagocyte function, focusing on neutrophils as the most important phagocytic cell type in the elimination of invading bacteria. Synthetic PSMs primed neutrophils for activation (as determined by neutrophil

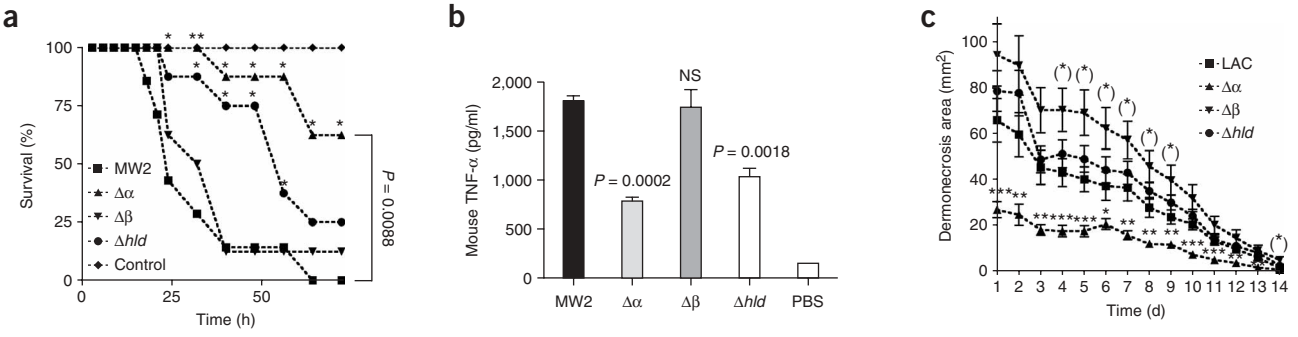

Figure 2 Mouse models of *S. aureus* infection. (**a**) Bacteremia model, survival curve. 10^8 CFUs of live *S. aureus* MW2 or isogenic PSM deletion strains in 0.1 ml sterile saline were injected into the tail veins of CD1 Swiss female mice ($n = 7$, wild-type; $n = 8$, all others). Control animals received sterile saline. Statistical analysis was performed with Fisher's exact test at each time point and the Kaplan-Meier test for survival curves (shown at the right). (**b**) Amount of TNF-α in mouse sera in the bacteremia model at the end of the experiment. The sera of each group were pooled and the TNF-α measurement was performed in triplicate. NS, not significant. (**c**) Skin and soft-tissue infection model. Crl: SKH1-hrBR mice ($n = 15$ for all groups) were inoculated with 50 μl PBS containing 10^7 CFUs of live *S. aureus* LAC, isogenic PSM deletion mutant strains, or saline alone as control, in the right flank by subcutaneous injection. Skin lesion area dimensions were measured daily with a caliper. (**d**) Photograph of representative lesions in mice 4 d after infection with the LAC wild-type and isogenic PSMα deletion strains. In **a**,**c**, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; (*), significant difference in the opposite direction (for Δβ strain, $P < 0.05$); all versus wild-type.

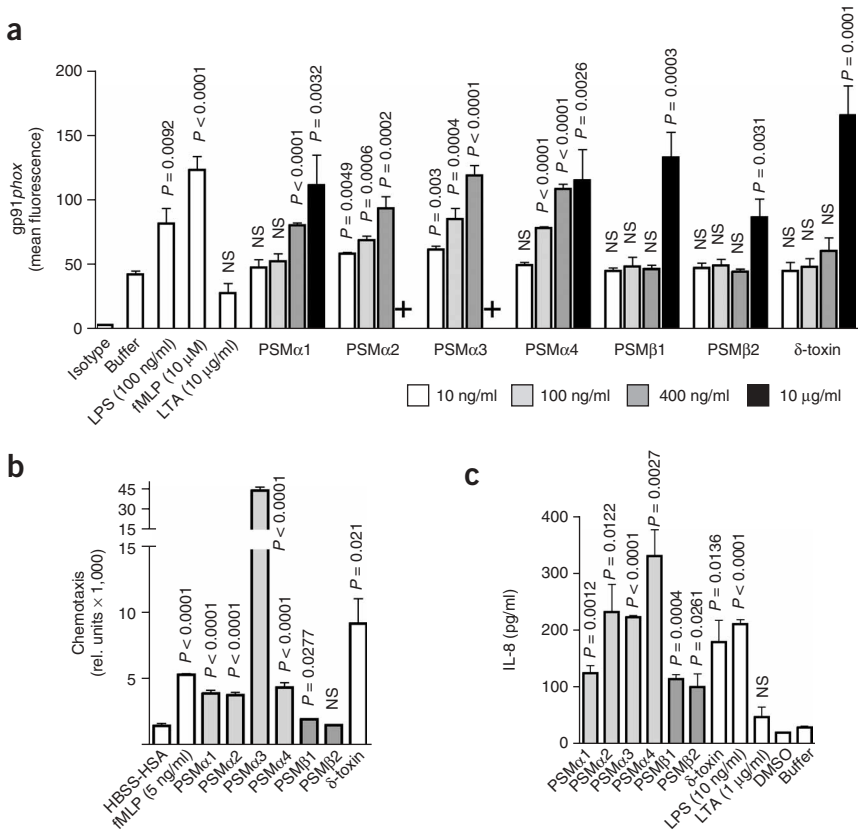

Figure 3 Interaction of PSMs with human neutrophils. (**a**) Surface expression of gp91*phox* after stimulation with synthetic PSM peptides (+, lysis of neutrophils occurred). *P*-values are versus buffer alone. (**b**) Chemotaxis after treatment with synthetic PSM peptides. PSM peptides were used at different concentrations (PSMα1, 5 μg/ml; PSMα2, 5 μg/ml; PSMα3, 500 ng/ml; PSMα4, 5 μg/ml; PSMβ1, 10 μg/ml; PSMβ2, 10 μg/ml; δ-toxin 2 μg/ml). To compare results, values were calculated for a theoretical concentration of 5 μg/ml. *P*-values are versus control experiment using HBSS with 0.05% human serum albumin (HBSS-HSA) (see **Supplementary Methods**). (**c**) Neutrophil secretion of IL-8 after treatment with synthetic PSM peptides at 10 μg/ml.

expression of gp91^{phox} and CD11b; **Fig. 3a** and **Supplementary Fig. 2** online), provoked neutrophil chemotaxis and Ca^{2+} flux (**Fig. 3b** and **Supplementary Fig. 2**) and induced release of the cytokine interleukin (IL)-8 (**Fig. 3c**), but not of tumor necrosis factor (TNF)-α or IL-1β. PSMα peptides, particularly PSMα3, generally showed the most pronounced proinflammatory activity. In contrast, PSMs did not increase the expression of IL-8, TNF-α or IL-1β in peripheral blood mononuclear cells or in Mono Mac 6 cells (data not shown). Thus, the proinflammatory activity of the PSMs is very specific, and the increase in TNF-α, a general indicator of inflammation, that we observed in the bacteremia model is probably a secondary effect of immune cell cross-activation. The changes in neutrophil IL-8 production that were observed after infection with the HA- and CA-MRSA wild-type, PSM deletion and PSM-complemented strains (for production levels of complemented strains, see **Supplementary Fig. 3** online) were consistent with the results obtained with synthetic PSMs (**Fig. 3c** and **Supplementary Fig. 2c,d**), indicating that α-type PSMs, particularly PSMα3, have a pronounced influence on the proinflammatory activity of CA-MRSA. Together, these results demonstrate that *S. aureus* PSM peptides efficiently activate human neutrophils, triggering an inflammatory response, and contribute greatly to staphylococcal virulence. As the enhanced virulence of CA-MRSA has been linked to leukolytic activity[14], we next used synthetic PSMs to determine whether PSMs lyse human neutrophils *in vitro*. Treatment with α-type PSMs, particularly PSMα3, caused substantial lysis (**Fig. 4a**). In accordance with this data, clarified culture media from CA-MRSA PSMα deletion strains and HA-MRSA strains had a greatly reduced capacity to induce lysis of human neutrophils (**Fig. 4b**). The lytic activity of these strains was entirely restored by genetic complementation with a plasmid expressing all α-type PSMs and was almost completely restored with a plasmid expressing PSMα3 alone, indicating that most of the noted cytolytic activity of CA-MRSA is caused by this peptide. In contrast, the PSMβ deletion and δ-toxin–negative strains did not show significantly reduced lysis of human neutrophils (**Fig. 4b**). Within 5 min of exposure to PSMα3, neutrophils showed signs of priming (such as flattening) and had formed structures that indicated that the integrity of the plasma membrane was compromised, as revealed by scanning electron microscopy (**Fig. 4c**). After 60 min, many neutrophils were completely destroyed. PSMs, mainly those of the α-type, also induced erythrocyte lysis, which may contribute to the development of disease (**Supplementary Fig. 4** online). Most probably, the membrane-damaging activity of PSMs is a result of their strong α-helicity (**Fig. 4d**) and amphipathy (**Fig. 4e**), which are typical features of pore-forming peptides[17]. Although *S. aureus* is known to produce factors that may cause neutrophil lysis *in vitro*, it is not understood which molecules are responsible for the elimination of neutrophils *in vivo*. In a mouse peritonitis model, neutrophil and monocyte infiltration and lysis were significantly increased after infection with CA-MRSA wild-type strains as compared to the isogenic PSMα deletion strains (**Fig. 4f** and **Supplementary Fig. 5** online). In addition, the PSMα-complemented HA-MRSA strain 252 induced significant increases in neutrophil and monocyte infiltration compared to the parental strain (**Fig. 4f** and **Supplementary Fig. 5**). These findings demonstrate that, to a large extent, the α-type PSMs are responsible for the enhanced cytolytic activity of CA-MRSA.

Collectively, our results indicate that a primary role of PSMs in pathogenesis is to destroy leukocytes, and thus PSMs are key players in the evasion of innate host defense by *S. aureus* (**Fig. 4g**). However, as PSMs also trigger the inflammatory response, it is crucial that the bacteria limit PSM secretion to times at which these immune cells can be efficiently inactivated. Of note, all *S. aureus* PSMs are tightly controlled by the *agr* quorum-sensing system (**Supplementary Fig. 6** online). This mechanism links gene expression to bacterial cell density through a secreted bacterial signal, limiting the expression of target genes to a time when the signal molecules reach a high concentration, such as when they are confined in the neutrophil phagosome[18,19]. Thus, it is presumably *agr*-mediated control that ensures that PSMs are produced when they are needed for pathogenesis and repressed when their production would jeopardize bacterial survival. Furthermore, strain-to-strain differences in PSM production

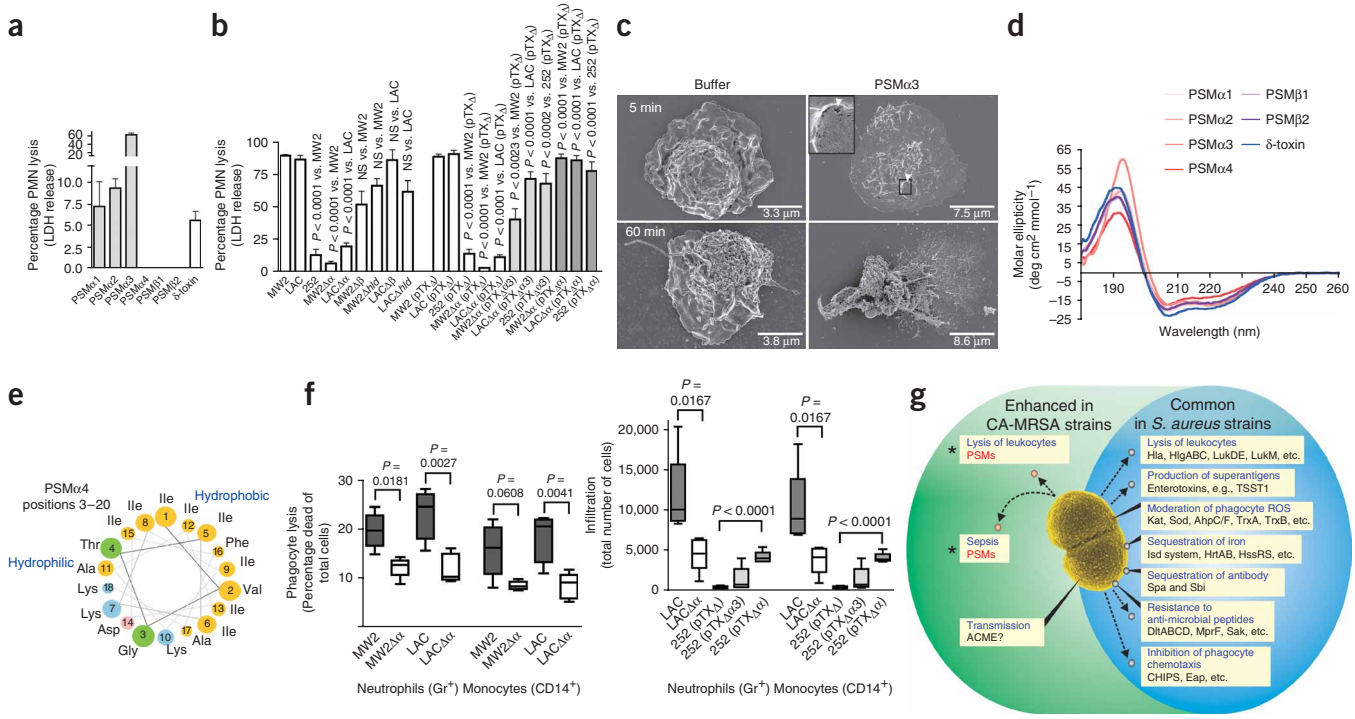

Figure 4 PSM-induced lysis of human neutrophils. (**a**,**b**) Human neutrophil lysis, as measured by release of lactate dehydrogenase (LDH) activity, after treatment with synthetic PSM peptides at 10 μg/ml (**a**) or with culture filtrates of wild-type, PSM gene deletion or complemented CA- and HA-MRSA strains (**b**). Data represent means ± s.e.m. of at least three independent measurements. (**c**) *In vitro* lysis of human neutrophils with synthetic PSMα3. Neutrophils were incubated with PSMα3 (10 μg/ml), observed by scanning electron microscopy at the time intervals indicated, and compared to buffer-treated controls. Arrowheads indicate structures interpreted as holes in the plasma membrane. (**d**) Circular dichroism spectra (taken in 50% trifluorethanol) of synthetic PSM peptides with *N*-formylmethionine. The peak at ~190 nm is indicative of α-helicity. (**e**) Helical wheel computation (http://cti.itc.virginia.edu/%7Ecmg/Demo/wheel/wheelApp.html) for PSMα4. (**f**) Infiltration and killing of human leukocytes in a mouse peritonitis model. Two hours after infection with bacteria, neutrophils and monocytes were counted in peritoneal exudates, and dead and live cells were distinguished by flow cytometry. $n = 5$ mice for all samples except MW2 samples, which had $n = 4$ mice (the results from one mouse in the MW2 wild-type and PSMα deletion strain samples were not included because of extensive bleeding). (**g**) Interaction of *S. aureus* with innate host defenses, and the basis for the increased virulence of CA-MRSA. *S. aureus* uses a series of mechanisms to evade host defenses (outlined on the right). The enhanced development of bacterial sepsis and leukocyte lysis induced by PSMs (*), as shown in this study, contributes to the exceptional virulence of CA-MRSA. Additionally, an increased capacity of CA-MRSA strains to colonize the host, which may be mediated in part by the arginine catabolic mobile genetic element (ACME)[5], might contribute to the success of CA-MRSA in causing human disease.

seem to be caused in part by differential *agr* activity. Production of RNAIII, the regulatory molecule of the *agr* system, was in general lower in HA-MRSA than in CA-MRSA strains. However, production of PSMs, especially of α-type PSMs, had limited correlation with RNAIII levels (**Fig. 1c**), suggesting that *agr*-independent regulation also contributes to the low PSMα abundance observed in HA-MRSA. These findings highlight the importance of understanding gene expression and regulation in the endeavor to determine the basis of CA-MRSA virulence.

Together, these results indicate that PSM peptides are major determinants of *S. aureus* virulence, and their increased production in CA-MRSA probably contributes to the enhanced virulence of those strains as compared to HA-MRSA[14]. Notably, the newly identified peptides encoded by the PSMα gene cluster have a significant effect on the ability of CA-MRSA strains to cause disease in animal infection models. By providing insight into the molecular basis of CA-MRSA virulence, this study advances the effort to find anti–CA-MRSA therapeutics and halt the epidemic spread of this human pathogen.

METHODS

Bacterial strains. HA- and CA-MRSA were standard strains whose genomes have been sequenced[4,5,20–22] and were obtained from NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*), or they were strains from a University of California, San Francisco collection representative of the leading sequence types isolated from human infections[23]. Bacteria were grown in tryptic soy broth (Becton Dickinson).

Construction of PSM and *agr* gene deletion strains and complementation plasmids. We constructed the PSMα and PSMβ deletion strains by allelic replacement with a spectinomycin-resistance cassette as described[24]. We constructed the *hld* deletion strains by altering the start codon of the *hld* gene using the procedure described[25] so as not to interfere with the function of RNAIII, in whose gene it is embedded[26]. The two PCR fragments used in that procedure were amplified with oligonucleotides that contained an *Mfe*I restriction site in place of the *hld* start codon, resulting in a one-base change (from ATG to ATT) and abolishing translation of *hld*. All PSM deletion strains were confirmed by analytical PCR with genomic DNA and RP-HPLC/ESI-MS of culture filtrates (**Supplementary Fig. 1**). *In vitro* growth of each deletion strain was indistinguishable from that of the corresponding wild-type strain (data not shown). In addition, we tested the secreted protein protease profiles of the *hld* deletion strains by SDS-PAGE and zymographic analysis[24] to ensure that the regulatory function of RNAIII was not affected (**Supplementary Fig. 1**). *agr* deletion strains were produced by phage transduction from strain RN6911.

The pTX_{Δ} plasmids were derived from plasmid pTX15 (ref. 27). We deleted the 5 part of the *xylR* repressor gene by digesting with *Nde*I and *Pst*I, then religating the plasmid. This allowed for high-level, constitutive expression

under control of the *xyl* promoter of genes cloned into the plasmid (**Supplementary Fig. 3**). The PSMα gene locus or the gene encoding PSMα3 were amplified by PCR using the chromosomal DNA of strain MW2 as template, digested with *Bam*HI and *Mlu*I, ligated into *Bam*HI- and *Mlu*I-digested pTX_{Δ} and transformed into *S. aureus* RN4220 and subsequently into the target strains. These plasmids confer resistance to tetracycline, which was added to growth cultures at a concentration of 12.5 μg/ml. *In vitro* growth of strains expressing the PSM-containing pTX_{Δ} derivatives was indistinguishable from that of the respective control strains (data not shown).

Mouse bacteremia, skin abscess and peritonitis models. Outbred, immunocompetent CD1 Swiss female mice and Crl: SKH1-hrBR hairless mice (Charles River Laboratories) were between 4 and 6 weeks of age at the time of use. These strains are widely used for the respective infection models[14]. *S. aureus* strains were grown to mid-exponential phase, washed once with sterile phosphate-buffered saline (PBS), then resuspended in PBS at 1×10^8 CFUs/100 μl (bacteremia model) or 1×10^7 CFUs/50 μl (abscess model) as described[12]. For the bacteremia model, we injected each mouse with 10^8 CFUs of live *S. aureus* in 0.1 ml sterile saline into the tail vein. Control animals received sterile saline only. After inoculation, mouse health and disease advancement were monitored every 3 h for the first 24 h, then every 8 h for up to 72 h. We euthanized the mice immediately if they showed signs of respiratory distress, mobility loss or inability to eat and drink. All surviving animals were euthanized at 72 h. At the time of death, serum samples were harvested from test animals.

For the abscess model, Crl: SKH1-hrBR mice were anesthetized with isoflurane and inoculated with 50 μl PBS containing 10^7 live *S. aureus* or saline alone in the right flank by subcutaneous injection. We examined test animals at 24-h intervals for a total of 14 d; we measured skin lesion dimensions daily with a caliper. We applied length (L) and width (W) values to calculate the area of lesions with the formula $L \times W$. All animals were euthanized after completion of the entire procedure.

For the peritonitis model, we injected CD1 Swiss female mice intraperitoneally with 0.1 ml PBS containing 10^7 live *S. aureus*. Two hours after the inoculation, we euthanized the mice with isoflurane and then injected 6.0 ml of RPMI medium containing 10% FBS into the abdominal cavities. Mice were surgically opened and 4.0 ml of exudates was collected with 23G needles. Aliquots of 0.4 ml of collected exudates were centrifuged at $500 \times g$ for 5 min and cell pellets were resuspended in 100 μl of staining buffer (PBS containing 1% goat serum). We stained the samples with FITC-conjugated antibody to mouse Ly-6G (clone 1A8, BD Biosciences), a neutrophil marker, with allophycocyanin (APC)-conjugated antibody to mouse CD14 (clone Sa2-8, eBioscience), a marker for monocytes and macrophages, or with appropriate isotype control antibodies. Propidium iodide (0.5 μg/ml, BD Biosciences) was used to identify dead cells. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) with CELLQUEST PRO software; data were collected for 20 s for each sample.

All mouse protocols were reviewed and approved by the Animal Use Committee at Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, US National Institutes of Health.

Accession codes. US National Center for Biotechnology Information: Coordinates for the PSM genes have been deposited with accession code BK006301.

Additional methods. For neutrophil-related methods, circular dichroism, electron microscopy, quantitative RT-PCR and statistical analysis, see **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank B. Diep and H. Chambers (University of California, San Francisco, USA) for *S. aureus* strains, T. Bae and O. Schneewind for plasmid pKOR1 (University of Chicago, Chicago, USA) and M. Teintze for help with circular dichroism measurements. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, US National Institutes of Health (to M.O. and F.R.D.) and the German Research Council (SFB 685) and the German Ministry of Education and Research (to A.P.).

AUTHOR CONTRIBUTIONS

M.O. isolated and identified the PSM peptides and performed HPLC/MS, D.K. performed chemotaxis experiments, R.W. and K.R.B. performed neutrophil lysis experiments, T.-H.L.B. performed bacterial growth curves, R.W., S.Y.Q. and M.L. performed animal experiments, R.W. and S.Y.Q. performed circular dichroism experiments, R.W. and A.D.K. performed flow cytometry, D.W.D. performed electron microscopy, and R.W. performed all other experiments. S.J.K., A.P., F.R.D. and M.O. supervised the experiments and M.O. prepared the manuscript.

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